Determinants of Sensitivity and Resistance to Rapamycin-Chemotherapy Drug Combinations In vivo

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Abstract

The phosphatidylinositol-3-OH kinase [PI(3)K] pathway is frequently activated in human cancers and represents a rational target for therapeutic intervention. We have previously shown that enforced expression of Akt, which is a downstream effector of PI(3)K, could promote tumorigenesis and drug resistance in the Eμ-myc lymphoma model, and that these tumors were particularly sensitive to inhibition of mammalian target of rapamycin (mTOR) with rapamycin when combined with conventional chemotherapy. We now show that reduced dosage of PTEN, a negative regulator of PI(3)K signaling, is sufficient to activate Akt, but has only a modest effect on lymphomagenesis in the same model. Nonetheless, loss of even one PTEN allele resulted in lymphomas that were resistant to conventional chemotherapy yet sensitive to rapamycin/chemotherapy combinations. These effects could be recapitulated by using RNA interference to suppress PTEN expression in lymphomas, which were previously established in the absence of PI(3)K lesions. Finally, the introduction of lesions that act downstream of mTOR (eIF4E) or disable apoptosis (Bcl-2 and loss of p53) into PTEN+/−/− lymphomas promoted resistance to rapamycin/chemotherapy combinations. Thus, whether activation of the PI(3)K pathway confers sensitivity or resistance to therapy depends on the therapy used as well as secondary genetic events. Understanding these genotype-response relationships in human tumors will be important for the effective use of rapamycin or other compounds targeting the PI(3)K pathway in the clinic. (Cancer Res 2006; 66(15): 7639-46)

Introduction

Tumorigenesis involves a series of genetic events that disrupt or alter signaling networks controlling proliferation and survival. The precise order of genetic alterations and their combinations that can confer malignant characteristics is variable, thereby producing heterogeneity in tumor behavior. As one example, increased oncogenic signals activate tumor suppressor programs, including apoptosis and senescence, and their disruption is an obligate requirement during tumorigenesis (1, 2). Disruption of apoptotic programs in tumor development can occur in different ways, for example through loss of tumor suppressor genes like ARF and p53, or increased activity of dominant oncoproteins like Bcl-2 (3) and survival pathways like the phosphatidylinositol-3-OH kinase [PI(3)K] pathway or its effectors Akt and eIF4E (4–6). Importantly, some of the same pathways that block apoptosis during tumorigenesis also impinge on the apoptotic response to chemotherapeutic drugs. Thus, the nature of the genetic lesions incurred during tumorigenesis to disrupt apoptosis can influence treatment behavior to varying degrees (4, 7–10). Conversely, strategies to restore apoptosis to tumor cells, either by increasing prosurvival signals, suppressing prosurvival signals, or by simultaneously achieving both, may prove effective for treating otherwise refractory tumors.

The PI(3)K pathway is implicated in cellular transformation and tumor development and contributes to the oncogenic activities of Ras and Bcr-abl [reviewed in ref. 11]. Concordantly, deregulation of this pathway is observed in many cancers, including lymphoma and leukemia, and most often involves inactivation of the negative regulator PTEN [refs. 12–14; reviewed in ref. 15]. Also, PTEN heterozygous mice develop tumors in multiple tissues, sometimes in the absence of complete PTEN inactivation, indicating that in certain contexts PTEN can be haploinsufficient for tumor suppression (16–19). Activation of the PI(3)K pathway has myriad effects on cellular physiology by virtue of its ability to regulate effectors controlling translation, metabolism, and cell survival (20–25). Although it seems likely that all of these properties contribute to tumorigenesis and drug resistance, the ability of deregulated PI(3)K signaling to promote cell survival seems particularly important (4).

Owing to its "gain-of-function" mode of action, the PI(3)K pathway represents an attractive therapeutic target, and compounds targeting multiple components of the pathway are in preclinical and clinical development (26). One drug that targets PI(3)K signaling is rapamycin, which acts to inhibit specific mammalian target of rapamycin (mTOR) complexes, thereby modulating translation in response to survival signals, or nutrient or energy availability. Initially approved as an immunosuppressant, rapamycin and its analogues have antitumor activity in some preclinical models and are currently in clinical trials (4, 27–32). It is therefore important to identify mechanisms of sensitivity and resistance to these agents.

We have previously described the effects of aberrant Akt expression on tumorigenesis, chemotherapeutic responses, and rapamycin sensitivity in the Eμ-myc lymphoma model (4). Specifically, we have shown that Akt dramatically accelerated myc-induced tumorigenesis and promoted resistance to conventional chemotherapy. Rapamycin suppressed mTOR activity and synergized with chemotherapy in Akt-expressing lymphomas, leading to potent antitumor responses. Interestingly, eIF4E, a translational regulator acting downstream of mTOR, accelerated lymphomagenesis and...
promoted drug resistance in a manner comparable with Akt, suggesting that a substantial portion of the Akt survival signal is transmitted through deregulated translation. Here, we studied the effects of reduced PTEN activity in the same genetic context and address the problem of rapamycin resistance, because this genetic lesion is the most prominent lesion that produces Akt activation in human cancers. We further evaluated genetic determinants of sensitivity and resistance cytotoxic agents, rapamycin, and combinations of both.

Materials and Methods

Generation of mice. Eμ-myc mice (C57BL/6 strain) and PTEN+/−, ARF−/−, p53−/− mice were crossed, and their offsprings were genotyped as described (17, 33). The animals were monitored for development of lymphoma and associated leukemia by biweekly palpation and blood counts, respectively. Upon the appearance of well-palpable lymphomas, the tumors were harvested and either fixed in formalin for histologic evaluation, or transfected with 10% DMSO, or transplanted in vitro culture (17).

Treatment studies. Treatment studies in mice were done as previously described (4). Briefly, 1 × 10^6 DMSO frozen or primary lymphoma cells were injected into the tail vein of 6- to 8-week-old female C57BL/6 mice. Upon the formation of palpable tumors, the animals were treated with rapamycin (4 mg/kg, i.p., 3×5 days), doxorubicin (10 mg/kg, i.p.), cyclophosphamide (300 mg/kg, i.p.), or combinations. In combination studies, the cytotoxic agent was given on day 2 of the rapamycin protocol. Rapamycin (LC Labs, Woburn, MA) was initially dissolved in 100% ethanol, stored at −20°C, and further diluted in an aqueous solution of 5.2% Tween 80 and 5.2% PEG 400 (final ethanol concentration, 2%) immediately before use. Doxorubicin (Sigma, St. Louis, MO) and cyclophosphamide (Sigma) were dissolved in water. In treatment studies, chemosensitive and chemoresistant control lymphomas were Arf-null lymphomas arising in a Eμ-myc/Arf−− background and p53-null lymphomas arising in a Eμ-myc/p53−− background, respectively (8). Treatment responses were monitored by twice weekly palpation and blood smears stained with Giemsa (Fisher Diagnostics, Middletown, VA). A "complete remission" was defined as the absence of any detectable tumor and leukemia. Tumor-free survival was defined as the time between treatment and reappearance of lymphoma or leukemia (4). Tumor-free and overall survival data were analyzed in the Kaplan-Meier format using the log-rank (Mantel-Cox) test for statistical significance.

Histopathology. Samples were fixed for 24 hours in 10% buffered formalin and embedded in paraffin. Thin sections (5 μm) were stained with H&E according to standard protocols. Detection of PTEN (99552, 1:100, Cell Signalling, Danvers, MA), phosphorylated Akt (9275, 1:100, Cell Signalling), phosphorylated ribosomal S6 protein (2215, 1:100, Cell Signalling), and Ki67 (1:100, NovoCastra, Newcastle upon Tyne, United Kingdom) was by standard avidin-biotin immunoperoxidase method, using biotinylated goat or rabbit specific immunoglobulins (Vector Labs, 1:100, Vector Labs, Peterborough, UK). Immunostainings were scored semi-quantitatively. As positive controls, sections from NOD/SCID (n = 3) and NSG (n = 2) mice were used. A 300× magnification was used for the quantification of the Ki67 index and a representative field was selected by visual inspection of consecutive slides. The number of tumor cells and leukemic cells in the center of the tumor were evaluated, and the percentage of Ki67-positive cells was calculated. The mean value and standard deviation were calculated for each animal. For each experiment, the data were analyzed using the log-rank (Mantel-Cox) test for statistical significance.
Burlingame, CA) at 1:500 and avidin-biotin peroxidase complexes (1:25, Vector Labs). Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. The apoptotic rate was analyzed by terminal deoxynucleotidyl transferase-mediated nick-end labeling assay (TUNEL) according to published protocols (34).

Fluorescence-activated cell sorting analysis. Tumor cell suspensions of representative tumors of each genotype were stained with the indicated monoclonal antibodies (PharMingen, San Diego, CA and CalTag, Burlingame, CA) conjugated with phycoerythrin, TRI-COLOR, or biotin, developed with streptavidin-allophycocyanin (PharMingen) and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest Pro software.

Western blotting. Immunoblots were done from whole-cell lysates as previously described (35). Fifty micrograms of protein per sample were resolved on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Antibodies against PTEN (a gift from M. Myers, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), phosphorylated Akt (9275, 1:1,000, Cell Signalling), Akt (9272, 1:1,000, Cell Signalling), ribosomal S6 protein (2212, 1:1,000, Cell Signalling), phosphorylated ribosomal S6 (2215, 1:1,000 Cell Signalling), and α-tubulin (B-5-1-2, 1:5,000, Sigma) were used as probes and detected using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ; Lumilight, Roche, Nutley, NJ).

Competition experiments. Tumor cells, either Eμ/myc/ARF−/− or Eμ/myc/PTEN+/−, were transduced with retroviral vectors at a low multiplicity of infection to create mixed populations of cells containing or lacking each vector. The vectors were MSCV-GFP (the empty vector control), MSCV-hel-2-IRES-GFP (10), MSCV-eIF4E-IRES-GFP (4), MSCV-abPTEN-SV40-GFP, MSCV-abPTEN-puro, MSCV-p53C (36), or MSCV-p53D (LMP-p53D, ref. 37). For in vitro experiments, the resulting mixed populations of infected and uninfected tumor cells were propagated in standard medium in the presence or absence of rapamycin (1 μM) and then analyzed for green fluorescent protein (GFP) content by flow cytometry. For the in vivo studies, the mixed populations were transplanted by tail-vein injection into nontransgenic female C57BL/6 animals; upon tumor formation, these mice were treated. The mice were typically sacrificed 48 hours later and single-cell suspensions of residual tumors were analyzed for GFP content.

Results

Given the high frequency of PTEN alterations in human tumors (15), we wished to address how PTEN heterozygosity would effect myc-driven tumorigenesis in vivo. To this end, we generated crosses of Eμ/myc to PTEN+/− mice and monitored the progeny for lymphoma development. Kaplan-Meier analysis comparing tumor latencies in Eμ/myc/PTEN+/− and their Eμ/myc/PTEN+/− littermates revealed only a modest acceleration of disease onset in the heterozygous animals that did not achieve statistical significance (P = 0.08; Fig. IA). Tumors in nontransgenic PTEN+/− animals were rare (data not shown). We analyzed DNA prepared from pure populations of tumor cells harvested from Eμ/myc/PTEN+/− animals by PTEN allele-specific PCR and found that the wild-type PTEN allele remained detectable in the majority of cases, with loss of heterozygosity only rarely observed (2 of 10 cases; Fig. IB).

Immunoblotting analysis on lysates from primary tumors confirmed that PTEN protein expression was retained in Eμ/myc/PTEN+/− tumors. Nonetheless, these tumors displayed increased ratios of phosphorylated to total AKT and ribosomal S6 proteins, suggesting that loss of one PTEN allele was sufficient to deregulate the PI(3)K pathway (Fig. 1C). PI(3)K pathway activation was, however, more varied and typically to a lesser extent than observed in tumors expressing an activated form of Akt (Fig. 1D; see also ref. 19). Similar results were observed in tissue sections examined by immunohistochemistry (Fig. 1E). Consistent with their similar tumor latencies, lymphomas arising in PTEN+/− and PTEN+/− animals showed similar rates of apoptosis and proliferation by TUNEL and Ki67 staining, respectively. Immunophenotypically, Eμ/myc/PTEN+/− tumors exhibited a mature B-cell phenotype, showing surface expression of B220 (CD45R), CD19, and surface immunoglobulin (data not shown). This immunophenotype was similar to that observed in Eμ/myc mice without loss of PTEN, but contrasts with the immature nonlinear determined phenotype of Eμ/myc lymphomas overexpressing AKT. Interestingly, over-expression of eIF4E, like PTEN loss, led to mature B-cell tumors (4). Thus, although loss of one PTEN allele can activate the PI(3)K pathway, its effect on lymphomagenesis is minimal. Because the PTEN+/− mouse is an embryonic lethal, we could not examine the effects of complete PTEN loss on lymphoma development using this model.

Given the minor effects of PTEN heterozygosity on tumor development, we wondered if this alteration would affect treatment response. We therefore transplanted primary tumors arising in Eμ/myc/PTEN+/− mice into multiple wild-type recipients. Upon tumor formation, we initiated therapy with either cyclophosphamide or doxorubicin and tracked its effect by biweekly tumor palpation and blood smears. Of note, in the absence of a cooperating oncogene (e.g., Akt or Bel-2) or preexisting loss of tumor suppressors (e.g., PTEN), myc-driven tumors are heterogeneous with ARF and p53 being each inactivated in ~25% of tumors (38). We therefore chose to compare PTEN+/− tumors with genetically defined Eμ/myc lymphomas lacking either ARF or p53, which are chemosensitive and chemoresistant, respectively (8). Doxorubicin treatment at the maximum tolerated dose of 10 mg/kg induced complete responses in 90% of mice bearing Eμ/myc/ARF−/− tumors. By contrast, the response of Eμ/myc/PTEN+/− lymphomas was significantly worse and approached the poor response of p53-null lymphomas, with only 50% of mice...
achieving a complete remission and none lasting longer than 3 weeks (Fig. 2A). Cyclophosphamide therapy at the maximum tolerated dose of 300 mg/kg is more effective against Eμ-myc lymphomas and induced complete remissions in all animals irrespective of tumor genotype (Fig. 2B). However, both Eμ-myc/ p53−/− and Eμ-myc/PTEN+/− tumors relapsed within 20 and 40 days, respectively, whereas half the animals bearing Eμ-myc/ ARF−/− tumors did not relapse in the 100-day observation period. Thus, lymphomas with loss of only one PTEN allele display a resistance to common chemotherapeutic agents that is comparable with loss of p53 or overexpression of Akt (4, 39).

In Akt-expressing tumors, rapamycin potently blocks mTOR activity in vivo and sensitizes these tumors to apoptosis induced by cytotoxic therapy (4). We therefore tested whether rapamycin would have similar effects in PTEN+/− tumors. Immunoblotting of lysates from Eμ-myc/PTEN+/− cells confirmed low levels of PTEN expression and subsequent activation of the PI(3)K/Akt pathway (Fig. 3A). These experiments also documented the ability of rapamycin to inhibit mTOR activity in vivo, as phosphorylation of ribosomal S6 protein was reduced 6 hours after rapamycin treatment relative to an untreated control. Doxorubicin did not affect S6 phosphorylation, and neither treatment affected phosphorylation of Akt (Fig. 3A). Immunohistochemistry on parallel tumors harvested 18 hours after rapamycin treatment revealed no further reduction in S6 phosphorylation compared with rapamycin alone, the tumors displayed substantially more apoptosis. Thus, rapamycin induces lasting mTOR inhibition and enhances the apoptotic response to DNA damage in Eμ-myc/PTEN+/− tumors.

We next asked whether the sensitization to apoptosis produced by rapamycin and chemotherapy would translate into a synergistic therapeutic effect in vivo. Indeed, cumulative analysis of “time-to-relapse” data from mice bearing Eμ-myc/PTEN+/− tumors revealed that the rapamycin/doxorubicin combination therapy induced complete remissions lasting at minimum 3 weeks and up to 45 days; by contrast, treatment with either drug alone achieved few responses and these were invariably short lived (rapamycin + doxorubicin versus rapamycin or doxorubicin: P = 0.0002; rapamycin versus doxorubicin: P = 0.5; Fig. 4A). The rapamycin/cyclophosphamide combination produced an even greater response and induced “cures” in almost 40% of animals (rapamycin + cyclophosphamide versus rapamycin or cyclophosphamide: P ∼ 0.02; rapamycin versus cyclophosphamide: P = 0.07; Fig. 4B). This effect was highly specific for tumors with an activated PI(3)K pathway in that treatment of chemosensitive Eμ-myc/ARF−/−−/− tumors with the same rapamycin and doxorubicin combination resulted in a reduced response compared with treatment with doxorubicin alone (P = 0.0002; Fig. 4C). Therefore, the synergistic antitumor activity of rapamycin-based combinations appears specific for tumors with PI(3)K/Akt pathway activating lesions. Surprisingly, sensitization to the effects of these agents requires loss of only one allele of PTEN.

These findings raise further questions regarding rapamycin sensitivity: (a) whether additional genetic lesions affect rapamycin sensitivity in PTEN+/− tumors and (b) whether it is important for PI(3)K activation to occur early in tumorigenesis to induce sensitivity to rapamycin. To address these issues, we established an in vivo competition assay. This approach allows us to generate genetically defined mixed tumor populations and examine the relative competitiveness of each subpopulation in vivo (Fig. 5A). Specifically, we infect a fraction of tumor cells from a defined genotype with a retroviral vector coexpressing a transgene or RNA interference (RNAi) construct together with GFP, creating a mixed

![Figure 3](image-url) **Figure 3.** Rapamycin inhibits mTOR activity in vivo and promotes apoptosis in response to chemotherapy. **A**, lysates prepared from Eμ-myc/PTEN+/− tumors either left untreated (U) or 6 hours following in vivo treatment with doxorubicin (D), rapamycin (R), or doxorubicin and rapamycin (D + R), and probed for PTEN, phosphorylated total Akt, and ribosomal S6 protein, and α-tubulin. **B**, representative micrographs of Eμ-myc/PTEN+/− tumors untreated or 18 hours after treatment with doxorubicin, rapamycin, or doxorubicin + rapamycin and stained with H&E or immunohistochemical probing detecting the phosphorylated forms of Akt and ribosomal S6 protein or TUNEL.
We therefore infected a subset of Eμ-myc/PTEN+/- tumor cells expressing eIF4E-/- tumor cells expressing eIF4E were also enriched following treatment with doxorubicin alone. Apparently, deregulation of translation beyond that produced by PTEN heterozygosity alone can further enhance chemoresistance (Fig. 5B, left).

Although PI(3)K lesions can contribute to sensitization to rapamycin therapy, the extent of this sensitization may be influenced by factors that act downstream (e.g., eIF4E) or in parallel to the oncogenic signaling pathway itself. For example, some studies suggest that rapamycin/chemotherapy combinations may require p53 tumor suppressor function, although the importance of p53 to rapamycin-based therapies seems variable (41–43). In our lymphoma model, mTOR inhibition, particularly in combination with chemotherapy, is associated with apoptosis induction, suggesting that an intact apoptotic program might be required for an optimal antitumor response. To determine whether p53 and/or apoptosis contribute significantly to the therapeutic responses in PTEN+/- tumors, we transduced short hairpin RNAs (shRNA) capable of suppressing p53 (36, 37), or a retrovirus overexpressing Bcl-2, into PTEN+/- lymphomas, and did in vivo competition assays. As expected, cells expressing p53 shRNAs were more resistant to doxorubicin, confirming the effectiveness of RNAi in this context (Fig. 5B, middle). More importantly, lymphoma cells expressing p53 shRNAs were also enriched following treatment with either rapamycin or combined treatment with doxorubicin and rapamycin, implying p53 function is also needed for optimal responses to these agents. Similar results were obtained in lymphoma populations harboring Bcl-2-expressing cells, which were more resistant to all three therapies (Fig. 5B, right; refs. 10, 44). Thus, genetic lesions acting downstream of mTOR or affecting parallel signaling networks can modulate rapamycin sensitivity in tumors in vivo.

In human cancer, PTEN inactivation is often a late event and associated with advanced disease (45). In the PTEN+/- tumors we studied, reduced PTEN expression and elevated PI(3)K signaling were presumably present during tumorigenesis (46). To determine whether acute ablation of PTEN in an established tumor could produce similar effects, we used RNAi to suppress PTEN function in ARF-deficient tumor cells, which show no evidence of PI(3)K pathway deregulation and are not sensitive to rapamycin. We generated a PTEN shRNA that could achieve a significant, albeit incomplete, knockdown of PTEN in pure populations of infected cells (Fig. 6A). Mixed populations of Eμ-myc/ARF-/- cells harboring this shPTEN construct or a vector control were treated with rapamycin in vitro. Seven days later, the cultures were subjected to flow cytometry to examine the percentage of GFP-expressing cells. Cells expressing PTEN shRNAs showed a marked competitive disadvantage, which was not observed in cells harboring the control vector (Fig. 6B). This implies that acute suppression of PTEN sensitizes cells to rapamycin, at least in vitro.

In parallel experiments, the mixed populations of the tumor cells described above were transplanted into recipient animals for in vivo treatment. Consistent with the ability of deregulated PI(3)K signaling to promote doxorubicin resistance (see Fig. 2A), tumor cells harboring PTEN shRNAs were enriched to near purity within 48 hours of doxorubicin administration (Fig. 6C). By contrast, treatment with rapamycin or the rapamycin/doxorubicin combination dramatically depleted the shPTEN-expressing cell population from mixed tumors in vivo. Thus, RNAi-mediated depletion of PTEN in established tumors can sensitize tumor cells to rapamycin-based therapies in a manner that is similar to tumors arising in an Eμ-myc/PTEN-/- background. These data also population of cells expressing or lacking the provirus. These populations are reintroduced into several recipient animals and, upon lymphoma manifestation, the tumor-bearing mice are either left untreated or treated with a drug or drug combination. Later, residual tumor cells are harvested and subjected to flow cytometry to determine the percentage of GFP-positive cells. Enrichment of GFP-positive cells following therapy (relative to the GFP fraction in untreated tumors) indicates that the construct enhances resistance to the therapy, whereas depletion of GFP suggests a sensitizing effect. Such internally controlled experiments provide a simple and sensitive assay to assess whether a particular lesion influences treatment response in vivo.

We first wished to determine whether deregulation of translational initiation downstream of mTOR would promote resistance to rapamycin/chemotherapy combinations in PTEN+/- tumors. eIF4e is a translation factor that is inhibited by 4E-BPs, which, in turn, are negatively regulated by mTOR-mediated phosphorylation (40). We therefore infected a subset of Eμ-myc/PTEN+/- tumor cells with a vector encoding eIF4E. As occurs in Akt-expressing lymphomas (4), eIF4E-expressing cells were enriched in PTEN+/- tumors 48 hours following rapamycin treatment or rapamycin/doxorubicin combinations (Fig. 5B, left), indicating that eIF4E enhances resistance to this drug combination. Furthermore, despite their initial poor response to conventional chemotherapy, PTEN+/- tumor cells expressing eIF4E were also enriched following treatment with doxorubicin alone. Apparently, deregulation of translation beyond that produced by PTEN heterozygosity alone can further enhance chemoresistance (Fig. 5B, left).
provide the proof of concept for the feasibility of in vivo RNAi screens to identify modulators of drug response in vivo.

**Discussion**

The PI(3)K pathway is activated in many tumors, for example, through mutations that target the PI3KA, PTEN, Akt, or TSC1 or TSC2 genes (reviewed in refs. 11, 47). PI(3)K activation can produce diverse changes in cell physiology and inhibits apoptosis, indicating that PI(3)K pathway mutations may have broad consequences on tumorigenesis and treatment responses. Owing to its gain-of-function mode of action, the pathway is also a compelling target for new therapeutics. Nonetheless, whether mutations in different components of the PI(3)K pathway produce the same effects on tumor physiology is not clear. We had previously described the effects of Akt expression on lymphomagenesis and treatment response in the Eμ-myc lymphoma model, and observed a dramatic effect of Akt in promoting tumorigenesis, resistance to conventional therapy, and sensitivity to rapamycin/chemotherapy combinations (4). However, lesions that activate PI(3)K signaling at the level of PTEN are more common in human cancer (15), and so we wished to determine how suppression of PTEN would affect tumor phenotypes in the Eμ-myc system.

Although our previous studies showed that Akt could dramatically accelerate myc-induced lymphomagenesis, Eμ-myc transgenic mice harboring loss of one PTEN allele showed little, if any, acceleration and most tumors examined retained the wild-type PTEN allele. However, the PI(3)K pathway is clearly activated in PTEN+/− lymphomas, although to a lesser and more varied extent than in tumors expressing an activated form of Akt (Fig. 1D). Likely, tumorigenesis in the presence of Myc requires a high flux through the Akt pathway (19). It remains possible, however, that high levels of Akt may have broader consequences for tumorigenesis than PTEN loss, either through "off-target" effects on other signaling networks or by differentially affecting various feedback loops. In this regard, the PTEN knockout mouse dies during embryonic development, precluding study of a complete ablation of PTEN on tumorigenesis in these animals. More recently, conditional PTEN knockout mice have been developed, and at least in a glioma model, complete loss of PTEN mimics Akt action during tumorigenesis (48).

Despite the relatively minor effects of PTEN heterozygosity on tumorigenesis, we noted marked responses on the response of
tumors to conventional or targeted therapy. Thus, loss of even one allele of PTEN was sufficient to produce chemoresistant tumors, a phenomenon also observed in lymphomas produced by over-expressing Akt. In principle, such resistance could represent a secondary consequence of other mutations arising in the PTEN+/− tumors, but we see that acute suppression of PTEN in an established tumor was sufficient to promote resistance to doxorubicin, and that rapamycin (which targets mTOR downstream of PTEN) restores drug-induced apoptosis to PTEN+/− tumors. Such a haploinsufficient effect of PTEN has been noted in tumorigenesis in a mouse prostate cancer model (19). Here, we see that reduced PTEN dosage can have a varied effect on tumor phenotypes, affecting treatment sensitivity to a greater degree than tumorigenesis in our model.

Mice harboring PTEN+/− lymphomas could be effectively treated using rapamycin/chemotherapy combinations, leading to durable responses that were similar to mice harboring Akt lymphomas. Also, as observed in mice harboring Akt lymphomas, mice with PTEN+/− lymphomas treated did not respond well to rapamycin therapy at our dose and schedule, despite the ability of the drug to efficiently inhibit mTOR activity. Nevertheless, previous studies indicate that PTEN loss can be sufficient to confer rapamycin sensitivity in some contexts (28, 29, 31), and we see that suppression of PTEN confers a selective disadvantage to tumor cells treated with rapamycin in culture. Thus, chemotherapy combinations are not required for rapamycin to have an antitumor effect, but potentiate the consequences of mTOR inhibition. Together, these data indicate that even modest changes in flux through the PI(3)K pathway can have profound effects on treatment behavior.

Figure 6. PTEN knockdown sensitizes established tumors to rapamycin in vitro and in vivo. A, immunoblot of lysates prepared from separate batches of Ba/F3/p210 cells transduced with either a control vector (vector; lanes 1-3) or an RNAi vector targeting PTEN (shPTEN; lanes 4-6), probed with antibodies against PTEN and tubulin as loading control. B, in vitro competition experiment. Summary of FACS analyses of GFP expression in E(λ)-myc/ARF−/− tumors partially transduced with an RNAI-GFP vector targeting PTEN (shPTEN) or a control vector-GFP (Vector) and either left untreated (white columns) or treated with 1 μmol/L rapamycin for 7 days (black columns), data are normalized to the untreated control (100%). Columns, mean; bars, SD. C, FACS analysis of E(λ)-myc/ARF−/− tumors partially transduced with an RNAI-GFP construct targeting PTEN (shPTEN) and harvested 48 hours after the indicated treatments.

Precisely how rapamycin and chemotherapy synergize to produce antitumor responses in E(λ)-myc lymphomas or other cancers remains to be determined, but our studies provide insights into its mode of action. One factor seems to be the ability of rapamycin to inhibit translation, as enforced expression of the translation initiation factor elF4E in PTEN+/− tumors enhanced resistance to chemotherapy or rapamycin/chemotherapy combinations. Another factor seems to be the presence of a PI(3)K pathway lesion, leading to the deregulation of PI(3)K signaling. Thus, tumors overexpressing Akt (4) or with reduced PTEN expression were sensitive to this combination and, at the same dose and schedule, ARF-deficient tumors [which show no evidence for PI(3)K involvement] showed a worse response to the combined therapy than to chemotherapy alone. Interestingly, these lesions did not have to be present during tumorigenesis, because acute suppression of PTEN in established tumors using RNAi could enhance rapamycin sensitivity.

Although a PI(3)K pathway lesion may confer sensitivity to rapamycin-based therapies, our data indicate that other pathways also influence rapamycin responses. Thus, disruption of p53 or overexpression of Bcl-2 in PTEN+/− tumors produced tumor cells that were more resistant to rapamycin/chemotherapy combinations. Interestingly, the role of p53 in influencing rapamycin responses is controversial, with studies differing on whether p53-deficient cells are more sensitive or resistant to rapamycin therapy in vitro (41–43, 49, 50). One
context. Understanding the precise molecular contexts whereby apoptosis mechanisms to rapamycin action may depend on the cellular of action, and conceivably the relative contributions of p53. However, these observations do not rule out p53-independent modes of action, and conceivably the relative contributions of p53 and apoptotic mechanisms to rapamycin action may depend on the cellular context.

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